The University of Alberta
Printing Department
Edmonton, Alberta

THE UNIVERSITY OF ALBERTA

CELL SURFACE CHANGES DURING DEVELOPMENT: MICROELECTROPHORESIS OF EARLY AMPHIBIAN EMBRYONIC CELLS

by

HARRIET LORENA HARRIS (nee MacMURDO)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1970

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Cell surface changes during development: Microelectrophoresis of early amphibian embryonic cells", submitted by Harriet Lorena Harris in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

In embryogenesis, cells undergo ordered movements during gastrulation. They lose existing contacts with their neighbours, migrate to new positions and form new contacts. Although it has been possible to trace the directional movements of these cells, the physical and chemical nature of their surface which may ultimately be related to cell repulsion and adhesion has never been fully determined. In an attempt to investigate the embryonic cell surface, cell electrophoresis was used to obtain information on the zeta potential and electrostatic charge. The purpose of this work was to study the mobilities of the presumptive germ layers; ectoderm, mesoderm and endoderm, and to follow their changes during development. This study represents an attempt to correlate physical-chemical changes at the cell surface to adhesion and repulsion occurring during morphogenetic movements of gastrulation.

Embryonic cells show higher electrophoretic mobilities than adult cells. Changes in surface charge density during development were found. Cells in midgastrula undergoing morphogenetic movements have the highest mobility, and therefore the greatest degree of cell repulsion. Sialic acids do not appear

to be involved in determining surface charge in these cells. These measurements are believed to be the first for early embryonic cells as well as the first for amphibian cells.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the support of Dr. S. E. Zalik throughout this project. Her guidance and advice, as well as her assistance in the preparation of the manuscript, were greatly appreciated.

I would also like to thank Dr. Saul Zalik for his help with the statistical analysis, Mrs. Carol Tilley for technical assistance, and my husband, Peter, for his encouragement throughout this study.

Financial support was provided by a Student bursary from the National Research Council, and a Teaching Assistantship from the Department of Zoology.

TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

INTRODUCTION AND LITERATURE REVIEW

One of the most complex problems in developmental biology is the mechanism of morphogenetic movements. These movements, occurring in all types of animal embryos, were first thoroughly described in amphibians (Vogt, 1929), probably due to their widespread accessibility and to the ease with which individual cells could be handled for cell dissociation and vital staining. Since these early studies, much work has been done on morphogenesis in echinoderms (Dan, 1960; Gustafson and Wolpert, 1963), chicks (New, 1959), and teleosts (Trinkaus, 1963), but the amphibian embryo remains a favourite material for experimental embryology.

The behaviour of dissociated cells of gastrulating embryos was first studied by Roux (1894) in *Rana fusca*. This was followed by an extensive study of gastrulation and cell movement using vital staining, by Vogt (1929). Studies on the mechanism of morphogenetic cell movements began with Holtfreter's (1939, 1943, 1944) work. This investigator was the first to describe in detail the processes of gastrulation in amphibian embryos and make extensive observations on the morphology and pattern of locomotion of migrating cells. He suggested that a cement-like material, which he called surface

coat, played a role in morphogenesis, wound healing, cell aggregation, and permeability.

Gastrulation involves cell deformation and cell movement, both of which are, to a certain extent, properties of the cell surface. Individual cells change their existing adhesive properties, undergo movements and form new contacts in predetermined positions. Some cells move as sheets of cells which maintain a certain degree of contact, while others move singly and exhibit amoeboid properties.

One of the methods used for studying the specificity of cell aggregation is by dissociating early embryonic tissues into individual cells. These cells tend to reassociate to form aggregates, and the distribution or sorting out of cells belonging to different tissues or germ layers can be subsequently studied. Amphibian embryos are particularly suited for these studies due to the ease of dissociation and aggregation of their cells.

Roux (1894) studying the behaviour of isolated cells in culture, first dissociated *R. fusca* embryos by using $Ca^{++}Mg^{++}$ free media. An effective method used by Holtfreter (1943) to dissociate amphibian embryonic cells was to raise the pH of the suspending medium. At pH 9.5 the cells dissociate, and upon

lowering the pH to 8.0 the cells spontaneously reaggregate. Chemical methods are now commonly used for obtaining cell suspensions. Chelating agents such as citrate (Feldman, 1955) and EDTA (ethylene diamine tetraacetic acid), which was introduced by Anderson (1953) and Zwilling (1954), are widely used at the present time. These agents appear to complex with divalent cations, in particular Ca^{++} and Mg^{++}, the presence of which are essential for adhesion (Steinberg, 1958). The use of trypsin has also been effective, particularly in avian and mammalian cells, and is generally combined with some mechanical agitation to separate the cells (Rous and Jones, 1916; Moscona and Moscona, 1952). Crude trypsin preparations are more effective than purified trypsin in producing single cells and are believed by some (Moscona, 1962) to work by digestion of extracellular material (ECM) which may cause cell adhesion.

From observations on tissue reaggregates, three basic models have been proposed for selective cell sorting out (Steinberg, 1964; Trinkaus, 1966): directed migration in response to a chemical stimulus (Townes and Holtfreter, 1958; Weiss, 1950; Moscona, 1962), a timing mechanism (Curtis, 1961) and differential adhesiveness (Steinberg, 1964). All models recognize the

existence of an initial non-specific adhesion which occurs at random between all cells of the reaggregate and a subsequent specific adhesion which only occurs between like cells and results in sorting out of groups of similar cells within an aggregate. The first of these models was based on the extensive work of Townes and Holtfreter (1955) on dissociation and subsequent reaggregation of cells from amphibian neurula embryos. These investigators made careful observations on ectoderm, neural plate, endoderm and mesoderm cells and their relative adhesive properties, and suggested that the sorting out of cells was in response to a chemical concentration gradient produced either in the medium or by the cells themselves. Individual cells, moving in response to these gradients would migrate either towards the surface or into the centre of the reaggregate. Two other models involving chemical substances have also been proposed. The first involves a mechanism for cell adhesion similar to antigen-antibody reactions and was proposed by P. Weiss (1950) based on the earlier work of Tyler (1940, 1942) on antibody-antigen type interactions at fertilization. This model was experimentally tested by Spiegel (1954, 1955) who obtained antisera against extracts of homogenized sponge cells. Partially purified antisera added to cell

reaggregating systems reversibly inhibited aggregation. Moscona (1960, 1962b, 1963) on the other hand believes cell adhesion is due to extracellular material composed most likely of mucopolysaccharides and other macromolecules. These substances are secreted by cells and form a cement which binds cells to one another or to a non-cellular substrate such as glass; it is species-specific and tissue-specific and allows for histogenetic reaggregation. The experimental basis for such a theory is primarily based on temperature effects (Moscona, 1961; Lilien and Moscona, 1967), inhibition of aggregation by RNase (Moscona, 1962b) and puromycin (Moscona and Moscona, 1965), and the dissociating action on tissues by trypsin (Moscona, 1952).

The timing mechanism put forward by Curtis (1961) takes into account the fact that cells may lose part of their cell surface adhesive properties during dissociation, particularly by chemical means. All cell types require time to recover from this effect but different cells will differ in the amount of time which elapses before they regain their adhesive properties. The first cells to do so will tend to become trapped at the surface, while the less adhesive cells will become trapped in the centre.

According to the model of differential adhesiveness,

(Steinberg, 1958, 1964), similar or like cells would contain more complementary binding sites on their cell surface than unlike cells and therefore would adhere more strongly. Initially, unlike cells would also form random non-specific adhesions but groups of like cells would sort out due to the stronger specific adhesions among them. Steinberg felt that those cells with the greatest number of complementary sites would form the strongest adhesions and would tend to migrate inwards in the reaggregate while those cells forming weaker adhesive bonds would come to lie on the outside.

Whatever the mechanism by which cells bind to each other, the chemical groups present on the cell surface must be involved to a certain degree in cell adhesion. The physical chemistry of cell adhesion has been studied in detail by both Pethica (1961) and Curtis (1966, 1967, 1969). The basis for this work rests in the Derjaguin-Landeau (1941) and Verwey-Overbeek (1948) theory of colloid stability. When applied to cells, it is known as the colloid electrostatic double layer theory of cell adhesion. This theory takes into account the electrical double layer present on the surface of all charged particles in suspension. That is, for every charge fixed on the surface of the particle, there is an opposite but equal charge in the liquid

around the particle. The distance between them is considered to be the thickness of the double layer. Pethica (1961) has listed the forces acting in cellular attraction and repulsion, including Van der Waals' forces which are responsible for weak attractions between molecules. The most important of the Van der Waals' forces that operate in cell adhesion is the London dispersion force, which is due to the interaction of dipoles acting between non-polar atoms at the cell surface. This force results in weak, short range attractive interactions between adjacent cell surfaces. Curtis (1960) believes that an intercellular gap of 150-200 Å is maintained by most cells in adhesion since closer approach is prevented by ionic repulsive forces due to like charges on the cell surface. If closer molecular contact is to occur, cells must overcome the repulsive barriers by high kinetic energy, as for example in tight junctions. Thus cell adhesion is due to a balance between repulsive forces and the dispersion force mentioned above occurring at a distance of 150-200 Å, which Curtis calls the secondary energy minimum. This gap is commonly found in electron micrographs (Robertson, 1959). Pethica (1961), however, believes that this intercellular gap might arise from fixation of the tissues, and

in fact cells do adhere in molecular contact. They overcome the repulsive barriers due to like surface charges by sending out projections less than 1μ in diameter (low radius of curvature projections). These projections initially come into contact with neighbouring cells, and secondarily longer stretches of the cell membrane may attach to each other. This investigator also feels that Van der Waals' forces are not sufficient to maintain cells in contact at this range, and therefore other forces of attraction are significant, such as bridging by Ca^{++} ions between adjacent acidic groups (Steinberg, 1958), and by large molecules, for example, proteins.

One of the techniques used to study the physical-chemical nature of the charged groups on cell surfaces is cell electrophoresis. Essentially, this technique involves the measurement of migration rates of cells in an electrical field. From measurements of electrophoretic mobilities (E.P.M.) the surface electrostatic charge and zeta potentials at the cell surface may be calculated. Two kinds of electrophoresis systems have been designed for this purpose. One type is the cytopherometer, initially used by Sachleben and based on earlier designs of rectangular measuring cuvettes by Abramson and Northrup and Kunitz (see Fuhrmann and

Ruhenstroth-Bauer, 1965). The second type of apparatus, and the one used throughout this study is the cylindrical cell electrophoresis apparatus first used by Bangham *et al.* (1958), and based on earlier designs by Ellis, Mitchell and Alexander and Saggers (See Seaman, 1965).

All cells so far measured have a negative electrophoretic mobility; that is, they migrate towards the positive pole. However, E.P.M. may vary widely between cells of different types and from different species of organisms. For example, rabbit erythrocytes move about half as fast as those of the human (Ruhenstroth-Bauer, 1965). The human erythrocyte has been studied to the greatest extent qualitatively as well as quantitatively; it has a mobility of 1.08 μ/v/sec/cm in 0.145 M NaCl (Seaman, 1965; Seaman and Heard, 1960). For this reason it is used in aligning the cell electrophoresis chamber. A great number of other normal cell types have been studied, including mouse and hamster fibroblasts (Forrester, 1962, 1964, 1965; Simon-Reuss *et al.*, 1964; Heard *et al.*, 1961), normal and regenerating rat liver cells (Ben-Or *et al.*, 1960), spleen cells (Forrester and Salaman, 1967) and bacteria (Douglas and Parker, 1958; Haydon and Seaman, 1962).

A number of studies have been performed on the relationship between electrophoretic mobility and the

development of malignancy among cells. Ambrose *et al.* (1956) showed that normal hampster kidney cells possess a lower electrophoretic mobility than homologous tumour kidney cells. Purdom *et al.* (1958) followed the development of mouse sarcoma cells and found that as they became malignant, they showed a corresponding increase in electrophoretic mobility. These authors felt that this increase in electrophoretic mobility was characteristic of cells undergoing malignant transformation, and it has since been shown that normal growth patterns can be restored by lowering the cell surface charge by basic substances (Ambrose, 1967, 1968). Ben-Or *et al.* (1960) showed that an increase in E.P.M. was also characteristic of normal liver cells undergoing regeneration, and concluded that this increase was generally shown by rapidly growing cells. Heard *et al.* (1961) measured embryonic mouse fibroblasts and found that they had a higher E.P.M. than the homologous adult fibroblasts.

A number of attempts have been made to show a correlation between electrophoretic mobility and adhesive properties of the cell surface. It is tempting to believe that the more negative the overall charge on the cell surface, the more these cells will repel each other and the less they will adhere. If

this were the case, it would be expected that cells with high E.P.M. would be less adhesive than cells with lower E.P.M.'s. When a change in adhesive properties in one cell type is followed, there is a corresponding change in surface charge. This applies to the change from normal to malignant cells (Purdom *et al.*, 1958) and the change from feeding to aggregating cells in the slime mould, *Dictyostelium discoideum* (Garrod and Gringell, 1970).

The negative electrophoretic charge of cells is a function of the ionogenic groups present on the cell surface. A great number of studies have been made in an attempt to identify the chemical groups responsible for this negative charge. Furchgott and Ponder (1941) suggested that the electrokinetic properties of cells were due to ionizable phosphate groups of the cell surface. In 1951, Ponder treated erythrocytes with trypsin and reported a 20% loss of negative charge. Cook, Seaman and Heard (1960) studied the effect of trypsin on erythrocytes and identified a sialomucopeptide containing carboxyl groups which was released by the enzyme, causing the loss in negative charge. They proposed that the sialomucopeptide N-acetylneuraminic acid formed a glycosidic link with D-galactosamine which in turn is linked to a trypsin susceptible

protein chain. The use of the enzyme neuraminidase to release the sialic acids by cleaving the α-glycosidic linkage was introduced by Cook, Heard, and Seaman (1961). Since this time, neuraminidase has been utilized to study a wide variety of mammalian cell surfaces, both normal (L. Weiss, 1941; Forrester *et al.*, 1962, 1964; Cook *et al.*, 1961, 1962) and malignant (Ruhenstroth-Bauer *et al.*, 1962; Langley and Ambrose, 1964; L. Weiss, 1965, 1968; Cook and Jacobson, 1968; Ward and Ambrose, 1969). On these cells, sialic acid has been found to contribute a significant proportion of the negative surface charges. Kemp (1968), using embryonic chick muscle cells recently found that neuraminidase inhibits cell aggregation. This study suggests that sialic acids may play a role in cell adhesion.

Although the ionogenic groups lost from the cell surface upon treatment with ribonuclease have not been identified, use of this enzyme also results in a decrease in the electrophoretic mobility of some cells, such as sarcoma, mouse liver and thymus cells (Mayhew and Weiss, 1968), Erlich ascites tumour cells (Weiss and Mayhew, 1966, 1967), but not of others, such as human and mouse erythrocytes (Mayhew and Weiss, 1968). RNase is also known to inhibit cell aggregation of

retinal cells from 7 day chick embryos (Moscona, 1962).

Although bacterial cells have a negative surface charge, their electrophoretic mobility is not affected by neuraminidase (Haydon and Seaman, 1962). Amino sugars have been postulated to account for the presence of carboxyl groups and therefore for the negative charge present on these cells (Davies *et al.*, 1956; Adams and Rideal, 1959; Haydon and Seaman, 1962), and amino acid decarboxylases decrease the negative charge of bacterial cell surfaces (James *et al.*, 1964).

It has been known for a long time that calcium, and to a lesser extent other divalent cations, play a role in cell adhesion and cell aggregation (Galtsoff, 1925; Steinberg, 1958, 1962; Curtis, 1960). The effect of calcium on the electrophoretic mobility and cell surface charge of dissociated heart and liver embryonic cells has been studied by Collins (1966). He found that the addition of calcium lowered cell E.P.M. and that heart cells, which are more cohesive, have greater calcium binding ability than liver cells, which are less adhesive. This evidence supports the calcium bridging theory argued by Pethica (1961) and Steinberg (1958).

Although electrophoretic mobilities have been measured for a variety of cells of adult and foetal

origin, no data are available on the electrokinetic and physical-chemical characteristics of the surface of early embryonic cells. It is of particular importance to study these cells, since they undergo ordered movements during gastrulation and changes in the nature of their surfaces may be reflected in changes in surface charge densities and consequently in their electrophoretic mobilities. No measurements have previously been reported for early embryonic cells, due to the difficulty in isolating a large cell population of each of the germ layers. In this study, discontinuous density gradient centrifugation was used to separate cells of the presumptive germ layers in embryos of the toad, *Xenopus laevis*, at the blastula, early gastrula, mid-gastrula and neurula stages. It was found that electrophoretic mobilities change during development, and are the highest when morphogenetic movements are occurring. These studies represent a first attempt to characterize the amphibian embryonic cell surface.

MATERIALS AND METHODS

A. Fertilization

Embryos of the toad, *Xenopus laevis*, were used. Eggs were obtained by injecting the animals with chorionic gonadotrophin (Antuitrin "S", Parke-Davis). Females were injected with 1000 international units (i.u.), males with 500 i.u. Frogs were paired immediately after injection and amplexus was allowed to occur in Brown and Caston's saline of the following composition:

NaCl	0.35 g
KCl	0.005 g
$CaCl_2$	0.01 g
$MgCl_2.6H_2O$	0.02 g
H_2O	1 litre

0.5 units/ml Penicillin-Streptomycin was added before use.

Fertilized eggs at the blastula stage were collected fourteen hours after injection, while early gastrula embryos could be obtained four to six hours later. Early blastula embryos maintained at 16°C developed into early or midgastrula 24 hours later, and into early neurula after forty-eight hours. In this way, successive stages of development could be used from

one egg clutch on three consecutive days.

B. Cell Dissociation

For each experiment, approximately one hundred eggs at the desired stage were placed for about ten minutes in a solution of 1% cysteine (Sigma Chemicals) - 1% papain (Calbiochem) in Steinberg's physiological salt solution (S.P.S.S.) of the following composition:

A. 17.0% NaCl

B. 0.5% KCl

C. 0.8% $Ca(NO_3)_2.H_2O$

D. 2.05% $MgSO_4.2H_2O$

Solutions of A, B, C and D were prepared as stock solutions and mixed in the following proportion:

A - 10 ml

B - 5 ml

C - 5 ml

D - 5 ml

distilled water - 500 ml

TRIS (hydroxymethylaminomethane) - 0.280 g

pH adjusted to 7.8-8.0 with 0.1 M HCl

The embryos were then transferred to 0.002 M EDTA in Ca^{++} Mg^{++} free Steinberg's pH 8.0, where the vitelline membranes were removed manually and eggs were allowed to dissociate. Gentle dissociation was accomplished

by placing the eggs in a rotating shaker in the cold. After one hour, cells were pipetted from the dissociating medium and washed in complete S.P.S.S. pH 7.8. Eggs which had not dissociated after this time were discarded.

C. Separation of Presumptive Germ Layers

Cells from each germ layer, ectoderm, mesoderm and endoderm were separated from one another by discontinuous density gradient centrifugation. Originally, density gradients of arabic gum in Stearn's saline were used but these proved inadequate because of the difficulty in preparing the arabic gum solution as well as cell damage caused by this compound. A much more suitable material was Ficoll (Pharmacia, Sweden) dissolved in S.P.S.S., pH 7.2. Gradients of Ficoll adequately maintained the embryonic cells in a healthy state throughout centrifugation. The densities of Ficoll ranged from 1.07 to 1.13 gm/ml for blastula cells and from 1.06 to 1.12 gm/ml for embryos at the gastrula and neurula stages. Gradients were formed in a 35 ml centrifuge tube by layering 5 ml of Ficoll solutions at each density successively from the bottom of the tube, beginning with the lightest density. Cells were then carefully layered on top of the tube and

centrifuged at 500 x g in an International Clinical centrifuge for ten minutes at 4°C.

For all stages studied, the result of this density gradient centrifugation was the formation of six bands. From microscopic observation of cell size, it appeared that the two lightest bands (A and B) contained presumptive ectodermal cells, the intermediate bands (C and D) mesodermal cells, and the two heaviest bands (E and F) presumptive endodermal cells. In order to test this, one hundred embryos were dissected into three respective regions, animal pole, marginal zone, and vegetal pole. Cells from each region were centrifuged in identical density gradients. The banding pattern of each germ layer essentially agreed with the microscopic observations. (See Results, Table 1).

In order to test the effect of Ficoll on cell viability, embryos were dissociated and centrifuged in the usual manner, except sterile conditions were maintained throughout. Following centrifugation, cells were incubated at 23°C for sixteen to eighteen hours in complete Steinberg's solution containing 0.5% human albumin (fraction V, Calbiochem). Small aggregates formed, both in cultures of centrifuged cell suspensions, and of control uncentrifuged cells.

Following centrifugation, each layer was pipetted

from the density gradient, washed, and resuspended in S.P.S.S., pH 7.2 for measurement of electrophoretic mobility.

D. Measurement of Electrophoretic Mobility

Originally, in order to compare measurements of electrophoretic mobility of these cells with those present in the literature, 0.145 M NaCl, pH 7.2 was used. However, S.P.S.S., pH 7.2, was found to preserve the health of the cells better than 0.145 M NaCl, and for this reason was used in all subsequent experiments. A comparison of the effect of these media on E.P.M. was carried out and appears in Results, Tables 3 and 5.

A cylindrical cell electrophoresis apparatus designed by Bangham *et al.* (1958) was used. It consists of an electrophoresis chamber made of a cylindrical glass tube into which platinum electrodes are inserted. It is placed into a thermostated water bath, maintained at 25°C in order to avoid thermal effects. The electrodes are connected to a power supply, and their polarity is reversed between measurements to guard against electrode effects which would result in movement due to drift. The electrodes were routinely platinized in an aqueous solution of 3% w/v platinum chloride and 0.03% w/v lead chloride, using a

six volt battery; the glass tube was routinely cleaned with alcoholic KOH. A microscope fitted with a water immersion objective lens is immersed in the water bath and focused on the optical flat surface of the glass tube. The apparatus was calibrated periodically with human erythrocytes from healthy donors kindly supplied by the Haemotology Department of the University of Alberta Hospital. Red blood cells suspended in 0.145 M NaCl, pH 7.2, have an electrophoretic mobility of 1.08± 0.03 (Seaman, 1965) and are commonly used as a standard.

The migration rate of the cells was measured by timing their passage over three squares of a graticule built in the microscope. The squares had been previously calibrated with a stage micrometer and measured 15μ each. Therefore each measurement represents the time taken by each cell to travel 45μ. The applied field strength was 4 volts/cm and the current was 0.8 amperes. Mobilities were expressed as microns/second/volt/cm. For calculations of zeta potential and surface charge density, the viscosity of the suspending media was measured with an Ostwald viscosimeter at 25°C±1°.

E. Neuraminidase Treatment

In order to determine the source of the negative charge present on the surface of these cells, tests

were performed with neuraminidase (Hoechst Pharmaceuticals). The enzyme treatment was adapted from Simon-Reuss *et al.* (1964) and was as follows: A 10 ml suspension of cells was washed in 0.145 M NaCl and centrifuged at 160 x g for five minutes. The supernatant was removed and the cells were divided into two groups. For the experimental group, one volume of cells was incubated with four volumes neuraminidase solution (20 i.u./ml in 0.145 M NaCl, 0.005 M $CaCl_2$, pH 7.2). In the control group one volume of cells was incubated with 3.2 volumes 0.145 M NaCl, 0.005 M $CaCl_2$, pH 7.2. After incubation for thirty minutes at 37°C, cells of both groups were centrifuged at 160 x g for five minutes. The supernatants of the experimental and control cells were removed and cells were suspended in 0.145 M NaCl, pH 7.2 for measurement of electrophoretic mobility. The control cell supernatant was reincubated at 37°C for thirty minutes with 0.8 volumes neuraminidase solution. Following this treatment, both supernatants were deproteinized with an equal volume of 10% trichloroacetic acid and, after centrifugation at 500 x g for two minutes, the precipitates were discarded and the supernatants assayed. In order to detect minute amounts of neuraminic acids which might be present, supernatants from experimental and control cells were

freeze-dried and each supernatant resuspended in 0.2 ml distilled water for measurement.

The amount of neuraminic acid released into the supernatant was determined by the thiobarbituric acid method (Warren, 1959). 0.2 ml of the supernatant was added to 0.1 ml of a 0.2 M sodium periodate solution. Tubes were shaken and left at room temperature for twenty minutes in order for oxidation to occur. One ml of a 10% sodium arsenite solution was added and shaken until all color dissappeared, and three ml of thiobarbituric acid solution was added. Tubes were then immersed in a boiling water bath for fifteen minutes, followed by incubation in the cold for five minutes. Cyclohexanone (4.5 ml) was added to each tube, shaken twice, and centrifuged for three minutes at 160 x g. The upper red cyclohexanone phase which contained the extracted chromophore was transferred to 3 ml cuvettes and the optical density at 549 mμ was read in a Beckman DU spectrophotometer. The optical density at 532 was also read, and subtracted from the values obtained at 549 mμ to correct for the contribution made by 2-deoxyribose. The amount of neuraminic acid present was calculated by the following formula: μm Neuraminic Acid = $0.09 \times O.D._{549} - 0.033 \times O.D._{532}$. This assay was performed on amphibian embryonic cells

and amphibian and human erythrocytes.

F. Adult and Embryonic Cells

A comparison was made between amphibian embryonic cells and amphibian adult erythrocytes to determine if the high electrophoretic mobility obtained for embryonic cells was characteristic of these cells only, or of amphibian cells in general. Amphibian erythrocytes were obtained from *Xenopus laevis* and *Rana pipiens* in a 10% EDTA solution. Cells were washed and resuspended in 0.145 M NaCl and their electrophoretic mobilities were measured.

RESULTS

A. Separation of Germ Layers

Density gradient centrifugation separated the embryonic cell population into six layers. The origin of these cells could be identified by the microscopic examination of size and presence of pigment granules. However, in order to further ascertain their identity, embryos of blastula and gastrula stages were dissected into the three presumptive germ layers, each of which was dissociated and centrifuged separately in identical gradients. The results are shown in Table 1. Cells of the animal pole region sedimented primarily in layers A and B, marginal zone cells in layers C and D, and vegetal pole cells in layers E and F. Some overlap is present, presumably due to the technique of micro-dissection.

The frequency distribution of electrophoretic mobilities for cells suspended in Steinberg's saline is represented in the histograms in Figures 1 to 4. In these graphs the rate of migration of individual cells over a forty-five micron distance is represented for the presumptive cell layers of the blastula (stages 8-9 Nieuwkoop and Farber, 1967), early gastrula (stage 10½), midgastrula (stage 11) and neurula (stage 12½)

embryos. An examination of these graphs show that mobilities follow an essentially normal distribution. Unimodality is also characteristic of each histogram; this argues for the presence of an essentially homogeneous cell population with respect to E.P.M. in each band. Frequency distribution histograms of cells suspended in 0.145 M NaCl have similar characteristics and have been reported elsewhere (MacMurdo and Zalik, 1970).

B. Electrophoretic Mobilities

The summarized data for a total of seventy-two hundred measurement of six layers at four stages are presented in Figures 1 to 4 and in Tables 2 and 3. These values were obtained over a number of experiments and represent an attempt to overcome the wide variation found between different clutches of eggs. Like all cells studied to date, amphibian embryonic cells show a negative electrophoretic mobility, however their values are higher than those found in the literature for other adult or foetal tissue cells. In order to determine whether the differences encountered between the presumptive embryonic layers and between developmental stages were significant, an analysis of variance by F and least significant difference (L.S.D.) tests

TABLE 1: Density gradient separation of cells obtained by dissociating different areas of embryos of *Xenopus laevis*. Microdissection was performed under a dissecting microscope with the aid of tungsten needles.

TABLE 1

A. BLASTULA CELLS

DENSITY	ANIMAL POLE	MARGINAL ZONE	VEGETAL POLE
Aqueous - 1.07	+		
1.07 - 1.08 (A)	++		
1.08 - 1.09 (B)	++		
1.09 - 1.10 (C)	+	++	
1.10 - 1.11 (D)		++	+
1.11 - 1.12 (E)			++
1.12 - 1.13 (F)			++

B. GASTRULA CELLS

DENSITY	ANIMAL POLE	MARGINAL ZONE	VEGETAL POLE
Aqueous - 1.06	+		
1.06 - 1.07 (A)	+		
1.07 - 1.08 (B)	++		
1.08 - 1.09 (C)	++	++	
1.09 - 1.10 (D)		++	+
1.10 - 1.11 (E)		+	++
1.11 - 1.12 (F)			++
Pellet			+

FIGURE 1: Frequency histogram of migration rates of blastula cells (stage 8 - 9). Migration rates are represented in seconds required to travel 45μ. Each histogram represents 300 measurements.

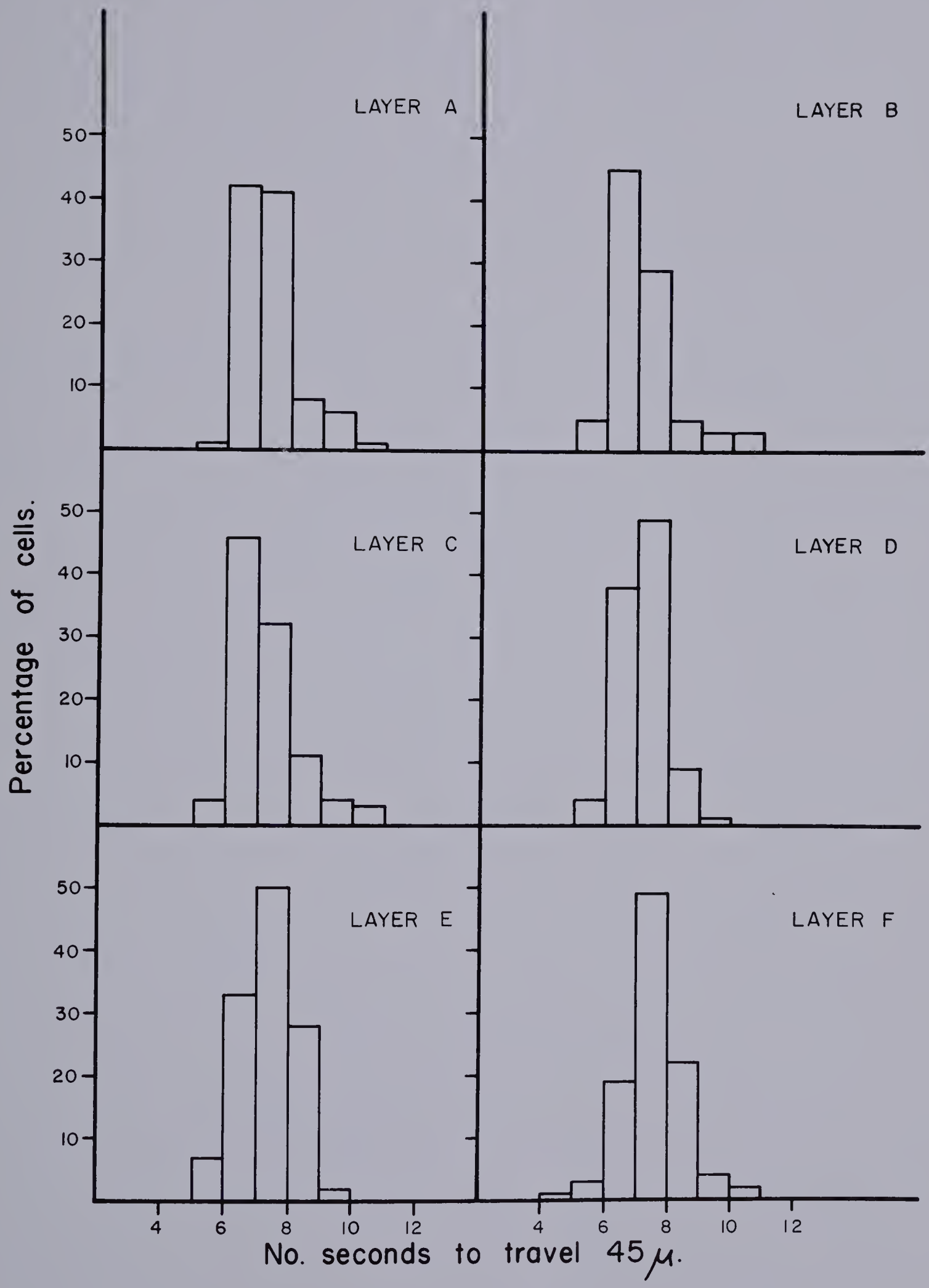

LAYER A
LAYER B
LAYER C
LAYER D
LAYER E
LAYER F
50
40
30
20
10
Percentage of cells.
4
6
8
10
12
No. seconds to travel 45 μ.

FIGURE 2: Frequency histogram of early gastrula cells (stage 10½). See Text, Figure 1.

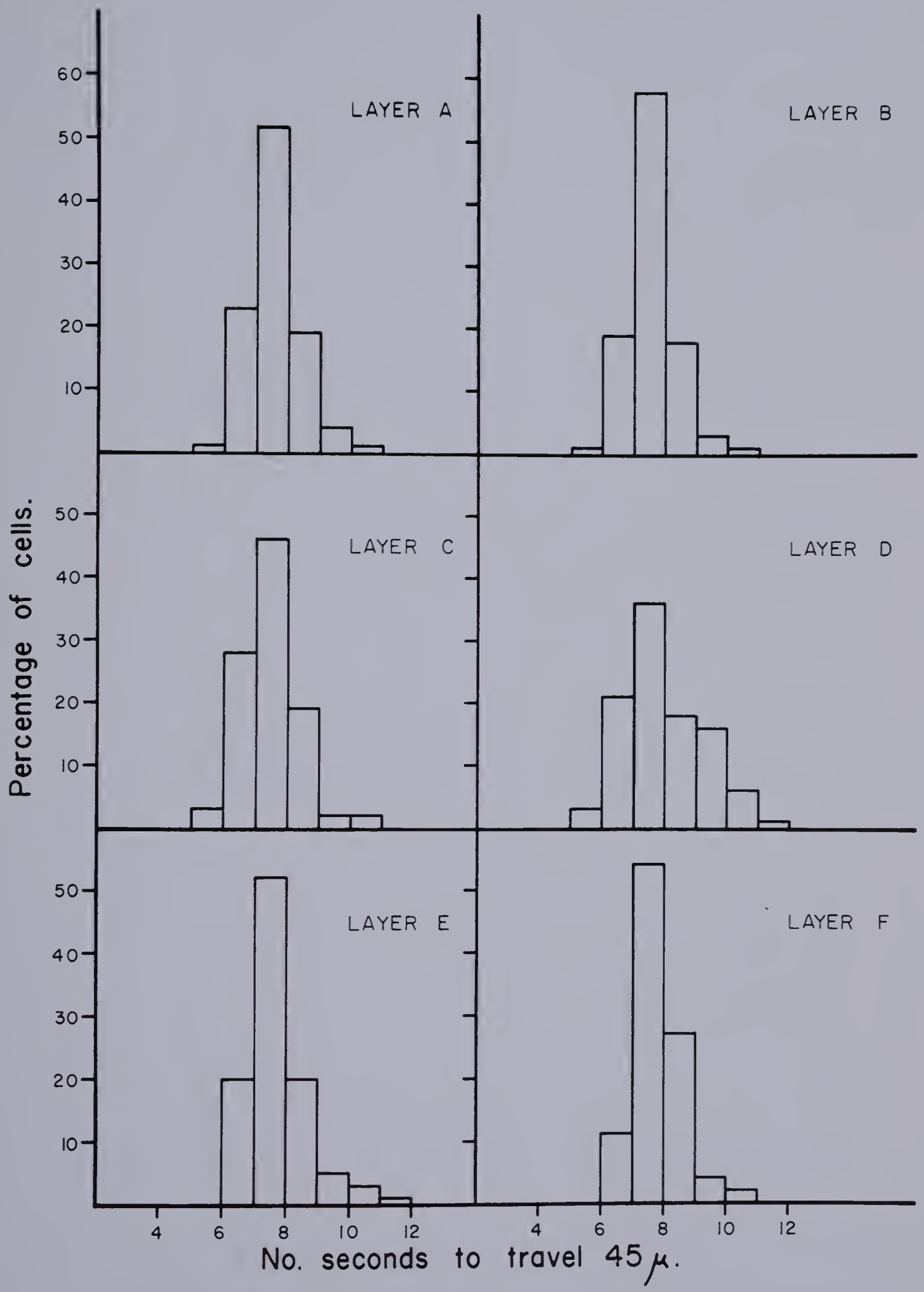

LAYER A
LAYER B
LAYER C
LAYER D
LAYER E
LAYER F
60
50
40
30
20
10
50
40
30
20
10
50
40
30
20
10
4
6
8
10
12
4
6
8
10
12
Percentage of cells.
No. seconds to travel 45 μ.

FIGURE 3: Frequency histograms for midgastrula cells (stage 11). See Text, Figure 1.

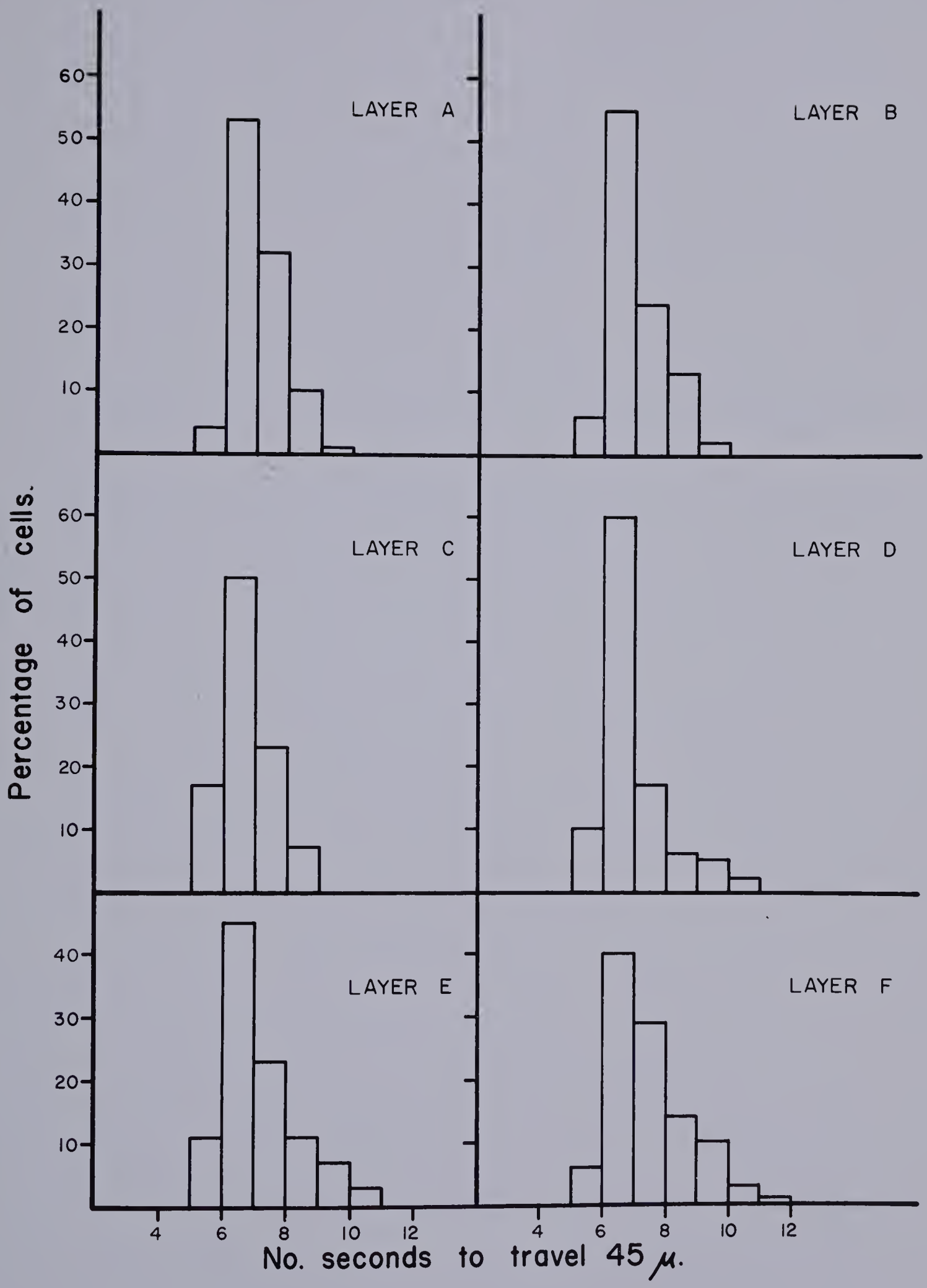
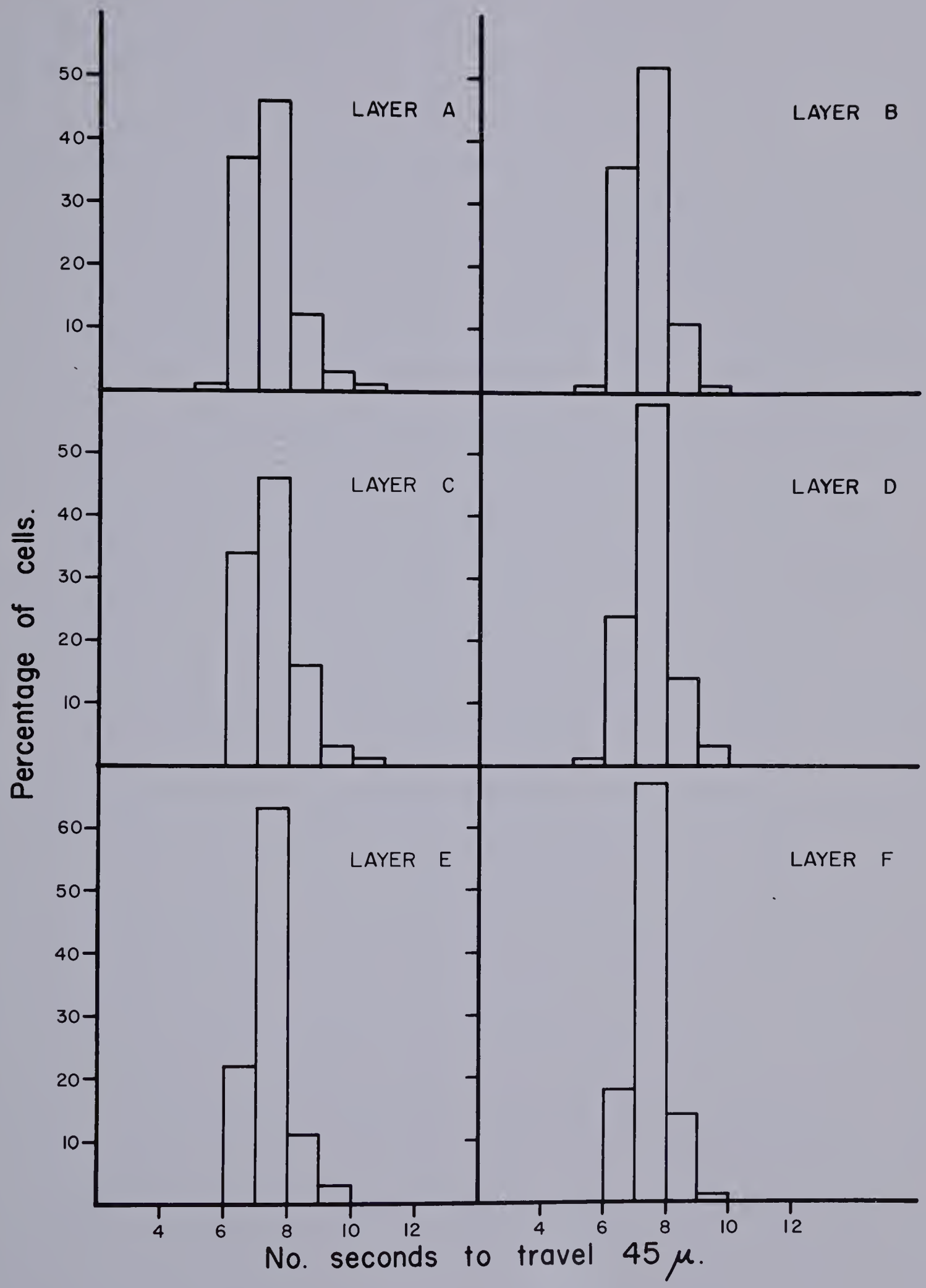

LAYER A
LAYER B
LAYER C
LAYER D
LAYER E
LAYER F
60
50
40
30
20
10
40
30
20
10
4
6
8
10
12
Percentage of cells.
No. seconds to travel 45 μ.

FIGURE 4: Frequency histogram for neurula cells (stage 12½). See Text, Figure 1.


LAYER A
LAYER B
LAYER C
LAYER D
LAYER E
LAYER F
50
40
30
20
10
60
4
6
8
10
12
Percentage of cells.
No. seconds to travel 45 μ.

were performed. The facilities of the University of Alberta Computer center were used to carry out these statistical analyses.

(a) Variation Between Stages

The analysis of variance of Table 2 indicates that differences between the four developmental stages studied are highly significant. Cells at early gastrula (mean migration rate 7.52 seconds) have a slower mobility than those of the blastula cells (mean 7.1 seconds) or cells of other stages. The most rapidly moving cells are those of mid-gastrula (mean 6.9 seconds); their mobilities decrease by neurula to an intermediate value (7.24 seconds) similar to that found at blastula. This difference between stages is consistent in all experiments. The cells with the lowest mobility at early gastrula are those of the mesodermal layer D (7.8 seconds) while the most rapid cell layer at midgastrula is the mesodermal layer C (6.6 seconds).

(b) Variation Between Layers

The overall variation between layers is not as great as that between stages and is significant only at the 5% level. Layers A (mean 7.14 seconds),

TABLE 2: Mean migration rates (expressed in seconds to travel 45μ). Each value is based on 300 measurements gathered from seven experiments.

TABLE 2

LAYERS	STAGES				MEAN
	BLASTULA	EARLY GASTRULA	MIDGASTRULA	NEURULA	
A	7.1	7.4	6.9	7.2	7.14
B	6.9	7.4	6.8	7.1	7.06
C	7.1	7.3	6.6	7.3	7.09
D	7.0	7.8	6.8	7.3	7.22
E	7.0	7.6	7.0	7.3	7.24
F	7.4	7.7	7.3	7.3	7.42
MEAN:	7.1	7.52	6.9	7.24	

Least significant difference at 1% level to compare stages is 0.2, layers 0.3 and the interaction 0.2.

ANALYSIS OF VARIANCE TABLE

SOURCE	DF	MS	F
STAGES	3	122.5	26.6**
LAYERS	5	20.8	4.5*
STAGES x LAYERS	15	4.6	5.6**
ERROR	7176	0.8	

* Significant at 5% level.

** Significant at 1% level.

FIGURE 5: Mean electrophoretic mobilities of presumptive germ layers during development.

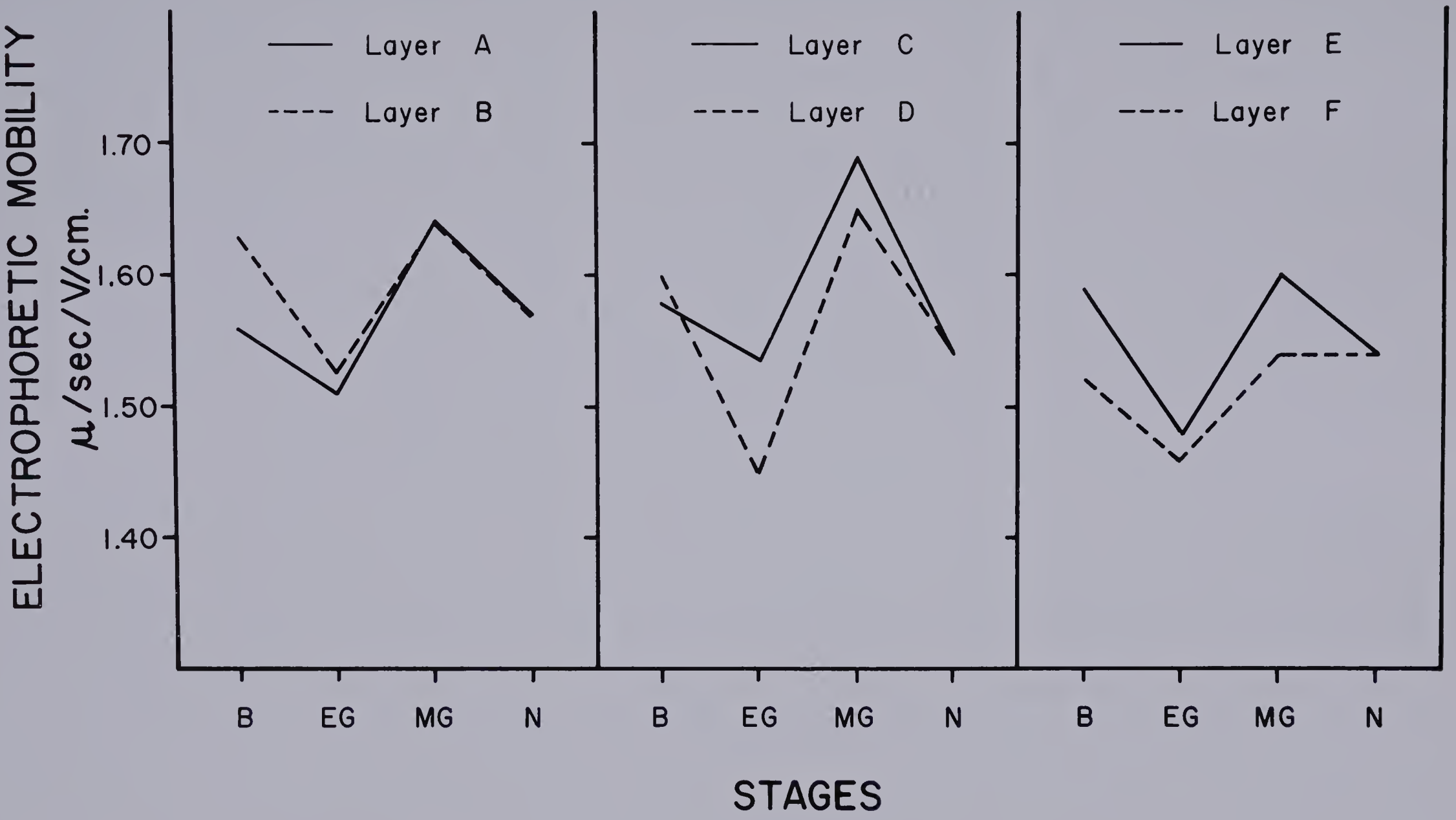
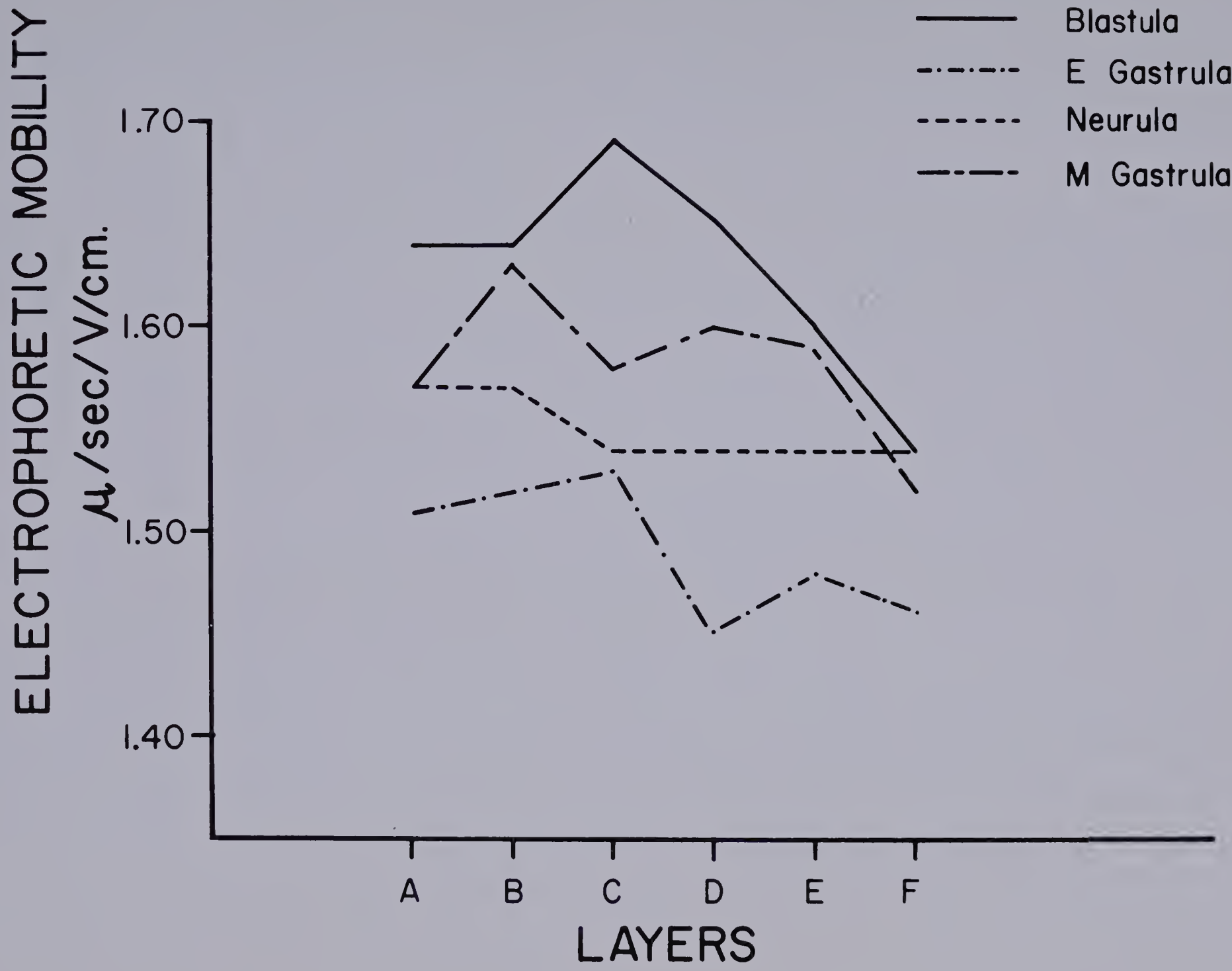

ELECTROPHORETIC MOBILITY
μ/sec/V/cm.
1.70
1.60
1.50
1.40
Layer A
Layer B
Layer C
Layer D
Layer E
Layer F
B
EG
MG
N
STAGES

FIGURE 6: Mean electrophoretic mobilities as a function of stage. This graph compares the rate of change for each layer as development proceeds.


ELECTROPHORETIC MOBILITY
μ/sec/V/cm.
1.70
1.60
1.50
1.40
A
B
C
D
E
F
LAYERS
Blastula
E Gastrula
Neurula
M Gastrula

B (mean 7.06 seconds) and C (mean 7.09 seconds) behave similarly; layer D (mean 7.22 seconds) and E (mean 7.24 seconds) are similar; while layer F is consistently slower than all other layers (mean 7.42 seconds). At the 1% level however, only ectodermal layers A and B and mesodermal cells of band C show a significant statistical difference from endodermal layer F.

(c) Interaction

When the individual layers are examined (Table 2), they all decrease in mobility between blastula and early gastrula, become more rapid at midgastrula and show intermediate values at neurula. However, the most significant change occurs in the mesodermal cell layers (C and D). These cells, particularly layer D, show the greatest decrease in mobility between blastula and early gastrula and the greatest increase between early gastrula and midgastrula (Table 2 and Figures 5 and 6). In contrast, the endodermal layers, especially layer F, show the least amount of change between stages. Thus it is the ectodermal cells, endodermal layer E and particularly the mesodermal cells which show a change in mobility as gastrulation proceeds. These characteristic

changes are shown in the graphs of Figures 5 and 6 in which actual mobilities in μ/sec/v/cm for layers and stages are represented.

By examining the data within the stages (Figs. 5 and 6), it can be seen that there is only slight change in mobilities for different layers of neurula, but considerable variation within each of the other stages. These variations in trends are reflected in the highly significant statistical differences for the interaction of Stages x Layers. At all stages, the ectodermal layers A and B are essentially alike, and the mesodermal layers C and D are similar, with the exception of early gastrula. However, the endodermal layers E and F are more variable.

Electrophoretic mobilities expressed in μ/sec/v/cm are presented in Table 3. In this table, the zeta potentials in millivolts (mv) and surface charge densities in electrostatic units (e.s.u./cm^2) for different embryonic layers are also indicated. These values have been calculated from the migration rates previously discussed and follow the same characteristic trends.

TABLE 3: Electrophoretic mobilities expressed as μ/sec/v/cm. These values have been calculated from the migration rates reported in Table 2. For calculation of zeta potentials and surface charge densities see text of Discussion.

TABLE 3

CELL TYPE	MOBILITY (μ/sec/v/cm)	ZETA POTENTIAL (mv)	SURFACE CHARGE DENSITY (e.s.u./cm²)
BLASTULA			
Layer A	1.57	26.69	4750
B	1.63	27.71	4952
C	1.58	26.90	4750
D	1.60	27.20	4849
E	1.59	27.03	4849
F	1.52	25.84	4551
E. GASTRULA			
Layer A	1.51	25.67	4551
B	1.52	25.84	4551
C	1.53	26.01	4650
D	1.45	24.65	4356
E	1.48	25.16	4453
F	1.46	24.85	4453
MIDGASTRULA			
Layer A	1.64	27.88	5050
B	1.64	27.88	5050
C	1.69	28.73	5150
D	1.65	28.05	5050
E	1.60	27.20	4849
F	1.54	26.18	4650
NEURULA			
Layer A	1.54	26.18	4650
B	1.54	26.18	4650
C	1.57	26.69	4750
D	1.57	26.69	4750
E	1.57	26.69	4750
F	1.57	26.69	4750

C. Variation Between Clutches

Since a wide variation was found in measurements between different clutches, cells of three separate egg clutches, each obtained from one male and female pair, were followed through successive stages of development. Electrophoretic mobilities appear in Table 4 and in Figure 7. Results tend to duplicate those obtained with a large number of measurements from different clutches. Cells show lowest mobilities at early gastrula and highest mobilities at midgastrula. This change in mobility is greatest for mesodermal cells of layers C and D, and least for the heavy endodermal layer F.

There are some differences found in these clutches. For example, in clutch 1 the E.P.M. of ectodermal layer A increases at midgastrula; however, its peak occurs at neurula. Ectodermal layers A and B of clutch 3 are similar. Nevertheless, the changes in mobilities follow the same trend as in the experiments mentioned in the earlier section.

D. Effects of Suspending Media on Electrophoretic Mobility

For purposes of comparison with data present in the literature, the medium originally used for measuring

FIGURE 7: Electrophoretic mobilities of presumptive germ layers from three clutches, each from a different female. Clutches were followed separately during development.

----- Experiments in which values for early gastrula were not available.

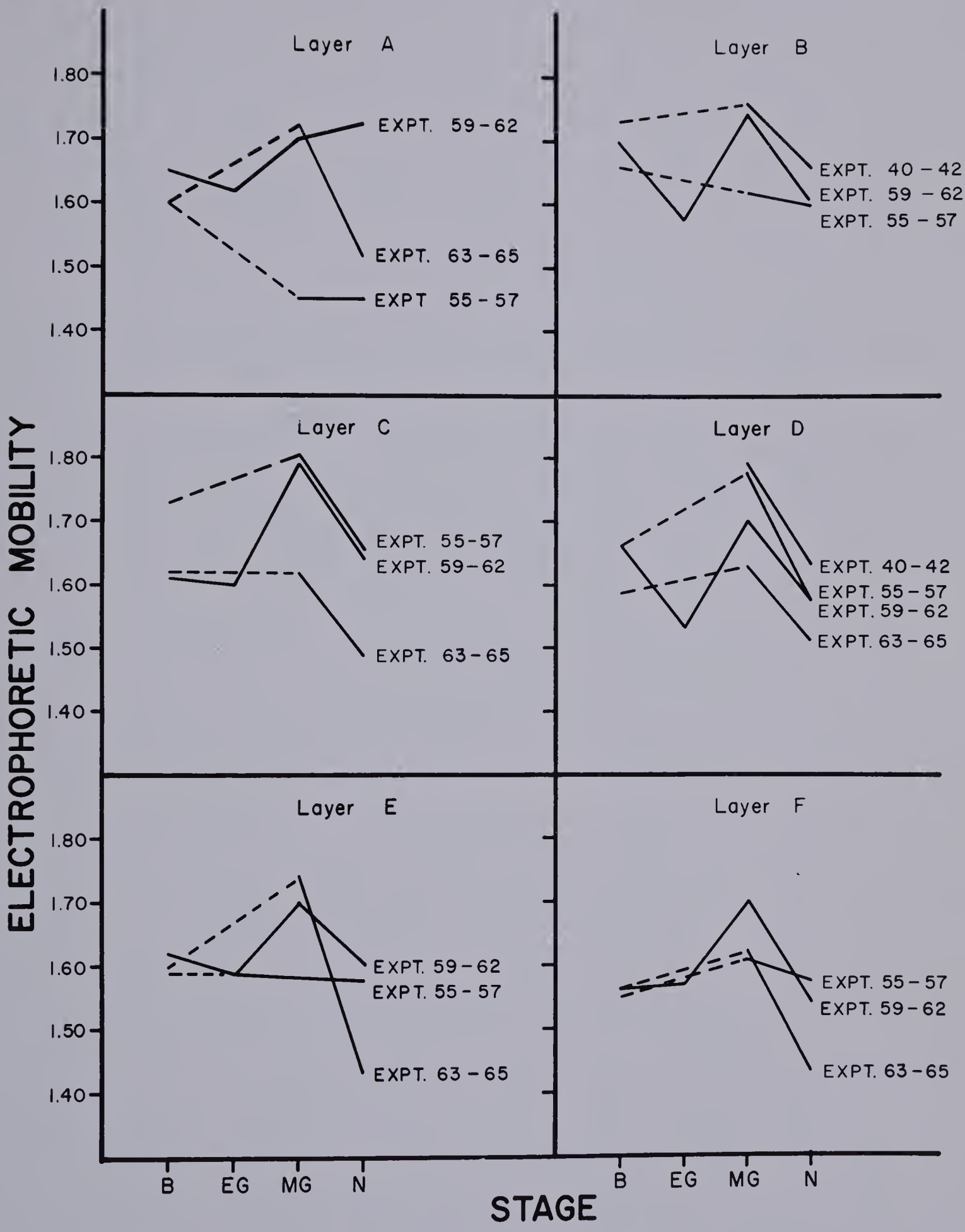

Layer A
EXPT. 59-62
EXPT. 63-65
EXPT 55-57
Layer B
EXPT. 40-42
EXPT. 59-62
EXPT. 55-57
Layer C
EXPT. 55-57
EXPT. 59-62
EXPT. 63-65
Layer D
EXPT. 40-42
EXPT. 55-57
EXPT. 59-62
EXPT. 63-65
Layer E
EXPT. 59-62
EXPT. 55-57
EXPT. 63-65
Layer F
EXPT. 55-57
EXPT. 59-62
EXPT. 63-65
1.80
1.70
1.60
1.50
1.40
B
EG
MG
N
ELECTROPHORETIC MOBILITY
STAGE

TABLE 4: Electrophoretic mobilities (in μ/sec/v/cm) of embryonic layers of egg clutches each derived from one female. Three clutches were followed through development. Each value represents an average of 50 cells.

TABLE 4

CLUTCH	STAGE	LAYER					
		A	B	C	D	E	F
1	Blastula	1.65	1.70	1.61	1.66	1.62	1.56
	Early Gastrula	1.62	1.58	1.60	1.53	1.59	1.57
	Mid Gastrula	1.70	1.74	1.79	1.70	1.70	1.70
	Neurula	1.72	1.61	1.64	1.57	1.60	1.54
2	Blastula	1.60	1.58	1.62	1.59	1.60	1.56
	Mid Gastrula	1.72	--	1.62	1.63	1.70	1.62
	Neurula	1.52	1.52	1.49	1.51	1.43	1.43
3	Blastula	1.60	1.66	1.73	1.63	1.59	1.55
	Mid Gastrula	1.45	1.62	1.80	1.78	1.59	1.61
	Neurula	1.45	1.60	1.65	1.57	1.58	1.57

TABLE 5: Electrophoretic mobilities of blastula cells measured in 0.145 M NaCl. The value for each layer represents an average of 350 measurements. For calculation of zeta potentials and surface charge densities see Discussion in the text.

TABLE 5

CELL TYPE	E.P.M. (μ/sec/v/cm)	ZETA POTENTIAL (mv)	SURFACE CHARGE DENSITY (esu/cm^2)
Layer A	1.38	23.87	6533
Layer B	1.38	23.87	6533
Layer C	1.31	22.66	6088
Layer D	1.27	21.97	5942
Layer E	1.38	23.87	6533
Layer F	1.38	23.87	6533

electrophoretic mobilities was .145 M NaCl pH 7.2 which has an ionic strength of 0.145. In order to obtain a better preservation of the cells, this was later changed to Steinberg's saline which has an ionic strength of 0.0735. Electrophoretic mobilities of blastula cells in 0.145 M NaCl are shown in Table 5. As expected, mobilities in .145M NaCl were lower than those measured in Steinberg's saline since a lower ionic strength increases cell electrophoretic mobility.

E. Electrophoretic Mobility in Adult Cells

Table 6 shows the electrophoretic mobility of adult amphibian erythrocytes. In contrast to early amphibian embryonic cells, erythrocytes of *Xenopus laevis* have an E.P.M. of 1.03 μ/sec/v/cm, and those of *Rana pipiens* have an E.P.M. of 1.05 μ/sec/v/cm. It appears that although the mobility of embryonic cells is characteristically high, some adult amphibian cells are, in fact, lower in mobility than the corresponding human cells. Therefore, high E.P.M. is not characteristic of amphibian cells but rather of early embryonic cells.

F. Neuraminidase Treatment

The results of the assay performed to test for the presence of N-acetyl neuraminic acid on the cell surface

TABLE 6: Electrophoretic mobilities of human and amphibian erythrocytes in 0.145 M NaCl, pH 7.2.

TABLE 6

ERYTHROCYTE	MOBILITY (μ/sec/v/cm)	STANDARD ERROR
1. Human	1.08	.008
2. *Rana pipiens*	1.05	.0015
3. *Xenopus laevis*	1.03	.011

TABLE 7: Effect of neuraminidase on the electrophoretic mobility of embryonic cells. For control purposes, erythrocytes of human and amphibian origin were used.

*Values represent mean electrophoretic mobilities and are expressed as μ/sec/v/cm.

TABLE 7

CELLS	TREATMENT	E.P.M.*	µm NEURAMINIC ACID RELEASED
Blastula	Control	1.67	0.0028
	Neuraminidase	1.63	0.0023
Erythrocytes (*Xenopus laevis*)	Control	0.98	0.0050
	Neuraminidase	0.99	0.0050
Erythrocytes (Human)	Control	1.08	0.0022
	Neuraminidase	0.36	0.0200

are shown in Table 7. Neuraminic acid has been repeatedly shown to be responsible to a large extent for the negative charge present on the surface of mammalian cells (Cook *et al.*, 1961, 1962; Forrester *et al.*, 1962, 1964). Control human erythrocytes showed a 66% decrease in E.P.M. concomitantly with the release of sialic acid when treated with neuraminidase. However, essentially no decrease in E.P.M. was observed and no neuraminic acid could be shown to be released from the surface of blastula cells or from amphibian erythrocytes. N-acetyl neuraminic acid does not appear to be responsible for the negative surface charge found on these cells.

DISCUSSION

The results obtained can be summarized as follows: (a) Early amphibian embryonic cells have high electrophoretic mobilities compared to other tissue cells, including adult amphibian erythrocytes. (b) There is a characteristic progressive change in mobility during gastrulation. Electrophoretic mobilities decrease significantly between blastula and early gastrula, increase to a peak at midgastrula, and decrease to an intermediate value at neurula. (c) The change in mobility between early gastrula and midgastrula is greatest for the mesodermal cell layers and least for the dense endoderm layer.

Cell electrophoretic mobility is a measure of the electrostatic charge present on the cell surface, and has been correlated with forces of attraction and repulsion acting between individual cells (Collins, 1966a,b; Pethica, 1961). These forces will, in turn, affect the ability of the cells to form adhesive contacts either with their neighbours or to a non-cellular substrate. If there is a direct correlation between electrophoretic mobility and repulsive properties of the cell surface, then the higher the mobility of a cell the less adhesive it will tend to be. On

this basis, it is not surprising to find embryonic cells exhibiting a high electrophoretic mobility since one would expect high repulsive forces resulting in loose adhesive contacts between cells of an early embryo. Such loose adhesion between embryonic cells is shown by their ease of separation. Cells at the blastula stage are only loosely associated and fall apart in $Ca^{++}Mg^{++}$ free media (Jones and Elsdale, 1963; Curtis, 1967; Landesman and Gross, 1968). Those of gastrula and early neurula are separated by treatment with a chemical agent such as ethylene diamine tetra-acetic acid. Cells of later stages become so closely associated that chelating agents no longer bring about their dissociation, and trypsin or other enzyme treatments must be used. Thus adhesive contacts between cells appear to increase as development proceeds.

Other cell types, however, are known to have high electrophoretic mobilities. E.P.M.'s of mammalian tissue cells fall in the range of 0.7 to 0.9, such as mouse fibroblasts which have a mobility of 0.82 μ/sec/v/cm (Heard *et al.*, 1961). Human erythrocytes, which are completely non-adhesive, are considered to have a high mobility (1.08 μ/sec/v/cm, Seaman, 1965). However, this varies widely for erythrocytes of different mammalian species (Abramson, 1942). Cells show an increase

in mobility when they undergo malignant transformations. This is associated with a decrease in their adhesive properties, and an increase in invasiveness (Ambrose *et al.*, 1956; Purdom *et al.*, 1958). Slime moulds, which change from a unicellular form to a multicellular aggregate, show a corresponding decrease in E.P.M. (Garrod and Gringell, 1970). On the other hand, rapidly dividing cells such as embryonic mouse fibroblasts (Heard *et al.*, 1961) and regenerating liver cells (Ben-Or *et al.*, 1960) have a high mobility. *Chlorella* cells, during logarithmic growth, have a higher mobility than those with a slow division rate (Lukiewicz and Korohoda, 1965). Mayhew (1966) has shown that dividing cells have higher electrophoretic mobility than those in the resting stage. This may be related to the preferential loss of adhesion to the substrate by mitotic cells (Robbins and Marcus, 1964). The high mobility shown by embryonic cells may, in part, be a result of their rapid division. However, gastrula cells have a low rate of division (Detlaff, 1964; Graham and Morgan, 1966) but show the greatest increase in E.P.M.

Forces of attraction and repulsion will not only influence adhesive characteristics but also affect the ease with which cells can move in relation to their neighbours. This is a particularly important

consideration for embryonic cells during gastrulation, since they undergo considerable movements *in vivo* which in turn may be related to cell surface phenomena (Weiss, 1965). It was for this reason that the germ layers were isolated and measured separately; it was expected that cells of germ layers which differ in the extent of their morphogenetic movements might also differ in cell surface charge.

Two ectodermal layers were isolated on the gradient. Cells of these two layers had similar mobilities at all stages tested, and they did not differ on the basis of cell electrophoresis. They had a consistently high mobility throughout. In the embryo, there are basically two types of ectodermal cells, the general ectoderm which will become the epidermis of the embryo, and the neural ectoderm which will form the neural plate and eventually structures of the central and peripheral nervous system. These two ectodermal cell types may or may not correspond to the two layers isolated by density gradient centrifugation. However, both move by the process of epiboly; a spreading out or expansion of the cells on the outside of the embryo. Thus these two cell types may be expected to show similar electrophoretic characteristics.

It is more difficult to correlate the two mesodermal

layers isolated to specific cell types present in the embryo. During gastrulation, head mesoderm, somite mesoderm, lateral mesoderm and tail mesoderm all invaginate through the blastopore and come to lie on the inside of the embryo after extensive morphogenetic movements. Presumptive notochordal cells are also included in these layers since they comprise a large proportion of the marginal zone of the pregastrula embryo (Nieuwkoop and Farber, 1967). Because of the heterogeneity of the mesodermal cell population, it is not possible to determine which cell type corresponds to a certain layer on the gradient. Nevertheless, the two mesodermal bands do not differ significantly in mobility at any stage other than early gastrula. These cells at midgastrula have the highest mobilities and show the greatest change between early gastrula and midgastrula. This high mobility is consistent with their large degree of movement in the embryo.

The two endodermal cell layers isolated differed considerably in terms of electrophoretic mobility. The less dense cell layer E was comparable to layers A, B, C and D, while the dense endoderm layer F was consistently slower than the others at every stage studied. In the amphibian embryo, there are two distinct endodermal cell populations; one, the head

endoderm, corresponds to the first cells that invaginate through the dorsal lip of the blastopore and eventually lie at the anterior end of the archenteron. They may correspond to the lightest endodermal band on the gradient, which has electrophoretic characteristics similar to the two mesodermal layers. The rest of the endodermal cells do not invaginate, but are merely covered with ectodermal cells, and will eventually form the floor of the archenteron. If there is a correlation between cell type in the embryo and cell type isolated on the gradient, then these cells most certainly correspond to the dense endodermal layer F, which has the lowest, most consistent electrophoretic mobility during development.

From the results of the foregoing experiments, and the data presented in Table 3, it is apparent that there is a significant change in cell surface charge density as cells undergo gastrulation. Blastula and neurula cells, although having high electrophoretic mobilities characteristic of embryonic cells, show intermediate values when compared to cells at other stages. Early gastrula cells have the lowest mobilities of all embryonic stages studied, while those at mid-gastrula have the highest E.P.M. In terms of repulsive interactions and adhesive ability, it may appear that

early gastrula cells would be comparatively less repulsive than those at midgastrula which tend to repel each other to the greatest extent. The mobility of early gastrula cells is nevertheless high enough compared to adult cells to account for a significant repulsive force between them. The difference in mobility between these two stages may be also explained by changes in the ease of cell deformability. Weiss (1965) has shown that a decrease in electrophoretic mobility may be related to an increase in deformability of the cell surface. It is known from Holtfreter's work (1943, 1944) and electron microscopic studies of Baker (1965) and those in our own laboratory (Sanders, 1970) that changes in cell shape occur at gastrulation, particularly during the migration of cells through the blastopore. It is, therefore, reasonable to suppose that early gastrula cells undergoing invagination will have lower mobilities and a greater tendency to elongate and change in shape. Midgastrula cells, most of which have moved through the blastopore and are migrating to their predetermined positions in the embryo, will have higher surface repulsive forces allowing for further cell migration. At neurula, when most of the cells are forming closer adhesions with their heighbours, electrophoretic mobility once again

decreases.

It is important to point out that the mesodermal layers show the greatest change in mobility between stages, and it is these cells which invaginate through the blastopore during early gastrula and migrate to the anterior region of the embryo during midgastrula. Cells which do not invaginate, the heavy endodermal cells, have the lowest most consistent mobility throughout the stages studied. Ectodermal cells show an intermediate degree of change between stages and, although they do not invaginate, undergo considerable cell movement during gastrulation.

Calculation of Zeta Potential and Surface Charge Density

Surface ionization and ion adsorption give particles in solution a characteristic charge. Electrophoretic mobility is a measure of these two processes. The zeta potential, which is the potential difference between the particle surface of shear and the bulk of the liquid, and the surface charge density, which depends on the composition of the surface and ionic constitution of the media, can be calculated from measurements of electrophoretic mobility (Abramson, 1942). These values are useful in comparing measurements of different cells in different media. The equation

used in this study for the calculation of zeta potentials is derived from the Helmholtz-Smoluchowski equation for charged particles in an electric field:

$$\mu = \frac{\zeta D}{4\pi\eta}$$

where ζ is the zeta potential, η is the viscosity of the suspending media, μ is the electrophoretic mobility and D is the dielectric constant of the medium (Abramson, 1942).

Thus, electrophoretic mobility is proportional to zeta potential but is only indirectly related to surface charge density. The surface charge (σ) in electrostatic units (e.s.u.) may be calculated by the equation of Gouy and Gorin (Abramson, 1942):

$$\sigma = \sqrt{\frac{NDKT}{2000\pi}} \sqrt{\Sigma C_i(e^{-Z_i\varepsilon\zeta/KT}-1)+ \Sigma C_j(e^{+Z_j\varepsilon\zeta/KT}-1)}$$

where N is Avogadro's number, D is the dielectric constant, K is the Boltzmann constant, T is the absolute temperature, C_i and C_j are the cations and anions in moles per litre, Z_i and Z_j are the valencies of the cations and anions, ε is the charge of the electron in e.s.u., and ζ is the zeta potential.

In this study, an approximation of the surface charge density was obtained by a simplification of this formula (James, 1965):

$$\sigma = 3.52 \times 10^4 \quad C \quad \sinh (\zeta/51.3)$$

where c is concentration in moles/l.

Although this equation is generally used with a suspending medium consisting of 1:1 electrolyte, it gives approximate values with media containing divalent cations when the total ionic concentration is considered. This may introduce a source of error, and results in approximate values of surface charge densities. Nevertheless, this error, if present, is constant for all cells measured in the medium used.

The following values were used to calculate the zeta potentials and surface charge densities reported in Tables 3 and 5: η, 1.05528 and 1.086059 centipoises for 0.145 M NaCl and Steinberg's saline respectively; D, 78.54, the dielectric constant of water was used in both media; C, 0.145 M and 0.61 M for NaCl and Steinberg's saline respectively.

Ionic strength of the suspending media inversely affects the measured electrophoretic mobility (Hunter, 1960). That is, as ionic strength increases, values of E.P.M. decrease. Thus it is not surprising that amphibian embryonic cells have a higher mobility in Steinberg's saline (ionic strength .0735) than in

0.145 M NaCl (ionic strength 0.145). According to Curtis (1967) ionic strength may alter electrophoretic mobility by "changing surface density and type of charged groups; the adsorption of small ions to the surface; modifying the thickness of the double layer; changing the type of macromolecules adsorbed to the surface; elution of membrane components and arrangement of surface components".

Ionic strength may affect the values of surface charge densities depending upon assmptions made in the equation used for their calculation. According to Hunter (1960) the surface charge density of human erythrocytes remains constant over a wide range of ionic strengths if the Gorin equation for the mobility of a charged spherical particle is used. However, the most common approach to calculate surface charge density is the flat plate equation of Gouy and Gorin which may introduce unknown error sources (Hunter, 1960). In order to compare the values obtained with those available in the literature, the simplification of the flat plate equation introduced by James (1965) was used in this study.

Amphibian cells were found to have a higher surface charge density in the medium with a higher ionic strength. This has also been found previously for human

erythrocytes (Furchgott and Ponder, 1941) and for tumour cells, which have a surface charge density of 3717 e.s.u./cm^2 in a medium with an ionic strength of 0.083 (Vassar, 1963) and 5400 e.s.u./cm^2 in a medium with an ionic strength of 0.150 (Forrester *et al.*, 1964, see Curtis, 1967). These values appear to agree with those obtained in this work.

This relationship is further complicated by the presence in Steinberg's saline of several anions and cations including calcium and magnesium while 0.145 M NaCl contains only sodium chloride. It is not possible from these experiments to separate the effect on mobility due to ionic strength from that due to the presence of the other ions in solution. By combining with the negative ionogenic groups of the cell surface, calcium and magnesium might be expected to lower electrophoretic mobility and surface charge density (Collins, 1966). This must be tested in a medium of the same ionic strength and must be investigated further. The presence of divalent cations, in particular calcium ions, is most likely responsible for the second effect of Steinberg's saline on electrophoretic mobility, that is, an alteration of the relative differences in mobility between layers. In 0.145 M NaCl, the mesodermal layers C and D were lower in

mobility than ectodermal (A and B) or endodermal (E and F), both of which have the same mobility. In Steinberg's saline at the blastula stage all layers vary in mobility to some extent. The greatest difference is seen between the ectodermal layer B, which has the highest E.P.M. (1.63 μ/sec/v/cm) and the endodermal layer F, which has the lowest (1.52 μ/sec/v/cm). This differential effect of ionic composition of the medium may reflect physical-chemical differences in the surface properties of the embryonic cell layers and requires further investigation.

In most mammalian normal and malignant cells so far studied, the carboxyl groups of N-acetyl neuraminic acid are responsible for a significant proportion of the negative electrophoretic mobility. However, no sialic acids could be detected on the cell surface of amphibian embryonic and adult cells. The release of large amounts of sialic acid from the surface of mammalian cells is accompanied by a decrease in electrophoretic mobility (Eylar *et al.*, 1962; Wallach and Eylar, 1961; Cook *et al.*, 1961). Therefore it is most likely that amphibian cell surfaces have no sialic acid since virtually none could be detected by either electrophoresis or spectrophotometry. Also, N-acetyl neuraminic acid does not appear to be responsible for

the negative charge present in *Amoeba proteus* (Sanders, 1969). Nevertheless, amphibian cells have a negative cell surface charge, which must be due to some predominant ionizable group or groups at the cell surface. Possible chemical groups that could account for this charge are carboxyl groups other than those found on sialic acids. These may be present in the sugar moiety of a mucopolysaccharide-type compound at the cell surface. Carboxyl groups of this type have been found to contribute significantly to the negative charge of bacterial groups (James, 1965) which do not contain sialic acids, and may be present on sheep polymorphonuclear leucocytes (Wilkins *et al.*, 1962). The presence of carboxyl groups can be demonstrated by treatment with diazomethane (Maccacaro and James, 1959; James, 1965) which esterfies the carboxyl groups with a decrease in electrophoretic mobility. Phosphate groups of lipids or nucleic acids may also be responsible for cell surface negative charge. Such charged groups have been postulated to account in part for the negative charge of lymphocytes and Erlich ascites cells (Bangham and Pethica, 1961). Further work must be done in order to characterize the chemical groups responsible for the negative charge present on the embryonic cell surface.

SUMMARY

1. Presumptive germ layers of early amphibian embryos from blastula to early neurula stages were separated by density gradient centrifugation and their electrophoretic mobility was measured.
2. The E.P.M. of early embryonic cells was found to be high compared to adult amphibian and mammalian cells.
3. Differences in E.P.M. were present between the presumptive germ layers. Mesodermal cells which show the greatest amount of movement in the embryo also show the greatest charge in mobility over the stages studied. Dense endoderm cells, which are the most stationery had the lowest and most consistent mobility.
4. Differences in E.P.M. were found between stages. Early gastrula cells had the lowest mobility of any stage while those at midgastrula had the highest mobility.
5. E.P.M. as well as zeta potential and surface charge density were found to be dependant upon ionic strength and composition of the suspending medium.

REFERENCES

ABRAMSON, H.A., MOYER, L.S., and GORIN, M.H. 1942. *In*: Electrophoresis of Proteins. (Ed.) Reinhold, New York.

ADAMS, D.M., and RIDEAL, E. 1959. The surface behaviour of *Mycobacterium phlei*. *Trans. Faraday Soc.* 85 : 185-189.

AMBROSE, E.J. 1967. *In*: The Proliferation and Spread of Neoplastic Cells, University of Texas, Houston. pp 23-39.

AMBROSE, E.J. and FORRESTER, J.A. 1968. *In:* Aspects of Cell Mobility, Cambridge University Press, Oxford. pp 237-248

AMBROSE, E.J., JAMES A.M., and LOWICK, J.H.B. 1956. Differences between the electrical charge carried by normal and homologous tumour cells. *Nature* 177 : 576.

ANDERSON, N.G. 1953. The mass isolation of whole cells from rat liver. *Science* 117 : 627-628.

BAKER, P.C. 1965. Fine structure and morphogenetic movements in the gastrula of the treefrog, *Hyla regilla*. *J. Cell Biol.* 24 : 95-116.

BANGHAM, A.D. FLEMANS, R., HEARD, D.H., and SEAMAN, G. V.F. 1958. An apparatus for microelectrophoresis

of small particles. *Nature* 182 : 642-644.

BEN-OR, S., EISENBERG, S. and DOLJANSKI, F. 1960. Electrophoretic mobilities of normal and regenerating liver cells. *Nature* 188 : 1200-1201.

COLLINS, M. 1966a. Electrokinetic properties of dissociated chick embryo cells. I. pH-Surface charge relationships and the effect of calcium ions. *J. Exp. Zool.* 163 : 23-37.

COLLINS, M. 1966b. Electrokinetic properties of dissociated chick embryo cells. II. Calcium ion binding by neural retinal cells. *J. Exp. Zool.* 163 : 39-47.

COOK, G.M.W., HEARD, D.H., and SEAMAN, G.V.F. 1961. Sialic acids and the electrokinetic charge of the human erythrocyte. *Nature* 191 : 44-47.

COOK, G.M.W., HEARD, D.H., and SEAMAN, G.V.F. 1962. The electrokinetic characterisation of the Ehrlich ascites carcinoma cell. *Exp. Cell. Res.* 28 : 27-39.

COOK, G.M.V., JACOBSON, W. 1968. The electrophoretic mobility of normal and leukaemic cells of mice. *Biochem. J.* 107 : 549-557.

COOK, G.M.V., SEAMAN, G.V.F., and HEARD, D.H. 1960. A sialomucopeptide liberated by trypsin from

the human erythrocyte. *Nature* 188 : 1011-1012.

CURTIS, A.S.G. 1960. Cell contacts: some physical considerations. *Am. Nat.* 94 : 37-56.

CURTIS, A.S.G. 1961. Timing mechanisms in the specific adhesions of cells. *Exp. Cell Res. Suppl.* 8 : 107-122.

CURTIS, A.S.G. 1966. Cell adhesion. *Sci. Progress* 54 : 61-86.

CURTIS, A.S.G. 1967. *In:* The Cell Surface, Logos Academic Press, London.

CURTIS, A.S.G. The measurement of cell adhesiveness by an absolute method. *J. Embryol. Exp. Morph.* 22 : 305-325.

DAN, K. 1960. Cyto-embryology of echinoderms and amphibia. *Intern. Rev. Cytol.* 9 : 321-367.

DETLAFF, T.A. 1964. Cell divisions, duration of interkinetic states and differentiation in early stages of embryonic development. *Adv. Morph.* 3 : 323.

DAVIES, J.T., and RIDEAL, E.K. 1961. *In:* Interfacial phenomena, Academic Press, London.

DERJAQUIN, B.V. and LANDEAU, L.D. 1941. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. *Acta*

Physiochemica URSS 14 : 633-662.

DOUGLAS, H.W. and PARKER, F. 1958. Electrophoretic studies on bacteria. 2. The effect of enzymes on resting spores of *Bacillus megaterium*, *B. subtilis*, and *B. cereus*. *Biochem. J.* 68 : 94-99.

EYLAR, E.H., MADOFF, M.A., BRODY, O.V., and ONCLEY, J. L. 1962. The contribution of sialic acid to the surface charge of the erythrocyte. *J. Biol. Chem.* 237 : 1992-2000.

FELDMAN, M. 1955. Dissociation and reaggregation of embryonic cells of *Triturus alpestris*. *J. Embryol. Exp. Morph.* 5 : 251-255.

FORRESTER, J.A. 1965. *In:* Cell Electrophoresis (ed.) E.J. Ambrose, J.A. Churchill Ltd., London.

FORRESTER, J.A., AMBROSE, E.J., and MACPHERSON, J.A. 1962. Electrophoretic investigations of a clone of hamster fibroblasts and polyoma transformed cells from the same population. *Nature* 196 : 1068-1070.

FORRESTER, J.A., AMBROSE, E.J., and STOKER, M.G.P. 1964. Microelectrophoresis of normal and transformed clones of hampster kidney fibroblasts. *Nature* 201 : 945-946.

FORRESTER, J.A., and SALAMAN, M.H. 1967. Cell electrophoretic mobilities in Friend virus disease. *Nature* 215 : 279-280.

FUHRMANN, G.F., and RUHENSTROTH-BAUER, G. 1965. *In:* Cell Electrophoresis. (ed.) E.J. Ambrose, J. and A. Churchill, Ltd., London, pp 22-25.

FURCHGOTT, R.F., and PONDER, E. 1941. Electrophoretic studies on human red blood cells. *J. Gen. Physiol.* 24 : 447-457.

GALTSOFF, P.S. 1925. Regneration after dissociation. (An experimental study on sponges). I. Behaviour of dissociated cells of *Microciona prolifera* under normal and altered conditions. *J. Exp. Zool.* 42 : 183-222.

GARROD, D.R. and GRINGELL, D. 1970. A progressive change in the electrophoretic mobility of pre-aggregation cells of the slime mould, *Dictyostelium discoideum*. *J. Cell Science* 6 : 277-284.

GOTTSCHALK, A. 1960. Correlation between composition, structure, shape and function of a salivary mucoprotein. *Nature* 186 : 949.

GRAHAM, C.F. and MORGAN, R.W. 1966. Changes in the cell cycle during early amphibian development. *Develop. Biol.* 14 : 439-460.

GUSTAFSON, T. and WOLPERT, L. 1963. The cellular basis of morphogenesis and sea urchin development. *Intern. Rev. Cytol.* 15 : 139-214.

HAYDON, D.A. and SEAMAN, G.V.F. 1962. An estimation of the surface ionogenic groups of the human erythrocyte and of *Escherichia coli*. *Proc. Roy. Soc. B* 156 : 533-549.

HEARD, D.H., SEAMAN, G.V.F., and SIMON-REUSS, I. 1961. Electrophoretic mobility of cultured mesodermal tissue cells. *Nature* 190 : 1009.

HOLTFRETER, J. 1939. *In:* Foundations of Experimental Embryology. (ed.) B. H. Willier and J. Oppenheimer, Prentice-Hall, 1964. pp 186-225.

HOLTFRETER, J. 1943. Properties and functions of the surface coat in amphibian embryos. *J. Exp. Zool.* 93 : 251-323.

HOLTFRETER, J. 1943. A study of the mechanics of gastrulation. Part I. *J. Exp. Zool.* 94 : 261-318.

HOLTFRETER, J. 1944. A study of the mechanics of gastrulation. Part II. *J. Exp. Zool.* 95 : 171-212.

HUNTER, R.J. 1960. Constancy of the surface charge density of human erythrocytes at different ionic strengths. *Arch. Biochem. Biophys.* 88 : 308-312.

JAMES, A.M. 1965. *In:* Cell Electrophoresis. (ed.) E. J. Ambrose, J. and A. Churchill Ltd.,

London, pp 154-170.

JAMES, A.M., LOVEDAY, D.E., and PLUMMER, D.T. 1964. Some physical investigations of the behaviour of bacterial surfaces. *Biochem. Biophys. Acta* 79 : 351-363.

KEMP, R.B. 1968. Effect of the removal of cell surface sialic acids on cell aggregation *in vitro*. *Nature* 218 : 1255-1256.

LANDESMAN, R. and GROSS, P.R. 1968. Patterns of macromolecular synthesis during development of *Xenopus laevis*. I. Incorporation of radioactive precursors into dissociated embryos. *Develop. Biol.* 18 : 571-589.

LANGLEY, O.K., and AMBROSE, E.J. 1964. Isolation of a mucopeptide from the surface of Ehrlich ascites tumour cells. *Nature* 204 : 53-54.

LUKIEWICZ, S., and KOROHODA, W. 1965. *In:* Cell Electrophoresis. (ed.) E.J. Ambrose, J.A. Churchill Ltd., London. pp 171-179.

MACCACARO, G.A., and JAMES, A.M. 1959. The irreversible lowering of the charge density of bacterial surfaces by diazomethane. *Biochem. Biophys. Acta* 36 : 279-280.

MACMURDO, H.L. and ZALIK, S.E. 1970. Embryonic cell surface: Electrophoretic mobilities of blastula cells. *Experientia* (in press).

MAYHEW, E. 1966. Cell electrophoretic mobility and

the mitotic cycle. *J. Gen. Physiol.* 49 : 717-725.

MAYHEW, E., and WEISS, L. 1968. Ribonucleic acid at the periphery of different cell types, and effect of growth rate on ionogenic groups in the periphery of cultured cells. *Exp. Cell. Res.* 50 : 441-453.

MITCHELL, P. 1948. A new microelectrophoresis cell. *Biochem. J.* 42 : 10.

MOSCONA, A.A. 1960. *In:* Developing Cell Systems and Their Controls. (ed.) D. Rudnick, Ronald Press, New York. pp 45-70.

MOSCONA, A.A. 1961. Effect of temperature on adhesion to glass and histogenic cohesion of dissociated cells. *Nature* 190 : 408-409.

MOSCONA, A.A. 1962a. Analysis of cell recombinations in experimental synthesis of tissues *in vitro*. *J. Cell Comp. Physiol.* (Suppl.) 60 : 65-80.

MOSCONA, A.A. 1962b. *In:* Biological Interactions in Normal and Neoplastic Growth. (eds.) M.J. Brennan and W.L. Simpson. Little, Brown and Co., Boston.

MOSCONA, A.A. 1963. Studies on cell aggregation: demonstration of materials with selective cell binding activity. *P.N.A.S.* 49 : 742-747.

MOSCONA, A. and MOSCONA, H. 1952. The dissociation and aggregation of cells from organ rudiments of the early chick embryo. *J. Anat.* 86: 287-301.

MOSCONA, M.H. and MOSCONA, A.A. 1965. Inhibition of cell aggregation *in vitro* by puromycin. *Exp. Cell Res.* 41 : 703-706.

NEW, D.A.T. 1959. The adhesive properties of expansion of the chick blastoderm. *J. Exp. Embryol Morph.* 7 : 146-164.

NIEUWKOOP, P.D., and FARBER, J. 1967. *In:* Normal Table of *Xenopus laevis*. North Holland Pub. Co., Amsterdam.

PETHICA, B.A. 1961. The physical chemistry of cell adhesion. *Exp. Cell Res.* (Suppl.) 8 : 123-140.

PONDER, E. 1951. Effects produced by trypsin on certain properties of the human red cell. *Blood* 6 : 350-356.

PURDOM, L. AMBROSE, E.J., and KLEIN, G. 1958. A correlation between electrical surface charge and some biological characteristics during the stepwise progression of a mouse sarcoma. *Nature* 181 : 1586-1587.

ROBBINS, E., and MARCUS, P.I. 1964. Mitotically synchronized mammalian cells: A simple method

for obtaining large populations. *Science* 144 : 1152-1153.

ROBERTSON, J.D. 1959. The ultrastructure of cell membranes and their derivatives. *Biochem. Soc. Symp.* 16 : 3-43.

ROUS, P. and JONES, F.S. 1916. A method for obtaining suspensions of living cells from the fixed tissues, and for the plating out of individual cells. *J. Exp. Med.* 23 : 549-555.

ROUX, W. 1894. Uber den "Cytotropismus" der Furchungszellen des grasfrosches (*Rana fusca*). *Arch. f. Entwickmech.* 1 : 43-68.

RUHENSTROTH-BAUER, G. 1965. *In:* Cell Electrophoresis. (ed.) E. J. Ambrose, J.A. Churchill Ltd. London. pp 66-72.

RUHENSTROTH-BAUER, G., FUHRMAN, G.F., KUBLER, W., RUEFF, F., and MUNK, K. 1962. Zur bedeutung der neuraminsauren in der zellmembran fur das wachstum maligner zellen. *Z. f. Krebsforsch* 65 : 37-43.

SANDERS, E. 1969. Studies on pinocytosis and the cell surface in amoebae. Ph.D. Thesis, U. of Southhampton, England.

SANDERS, E. 1970. Personal Communication.

SEAMAN, G.V.F. 1965. *In:* Cell Electrophoresis. (ed.)

E.J. Ambrose, J. and A. Churchill Ltd. London. pp 4-21.

SEAMAN, G.V.F. and HEARD, D.H. 1960. The surface of the washed human erythrocyte as a polyanion. *J. Gen. Physiol.* 44 : 251-263.

SIMON-REUSS, I., COOK, G.M.V., SEAMAN, G.V.F., and HEARD, D.H. 1964. Electorphoretic studies of some types of mamalian tissue cell. *Cancer Res.* 24 : 2038-2043.

SPIEGEL, M.J. 1955. The reaggregation of dissociated sponge cells. *Ann. N.Y. Acad. Sci.* 60 : 1056-1078.

SPIEGEL, M.J. 1954. The role of specific antigens in cell adhesion. *Biol. Bull.* 107 : 130-155.

STEINBERG, M.S. 1958. On the chemical bonds between animal cells: a mechanism for type specific association. *Am. Nat.* 92 : 65-82.

STEINBERG, M.S. 1962. *In:* Biological Interactions in Normal and Neoplastic Growth. (eds.) M.J. Brennen and W.L. Simpson. Little, Brown and Co., Boston.

STEINBERG, M.S. 1964. *In:* Cellular Membranes in Development. (ed.) M. Locke. Academic Press, New York. pp 321-366.

TOWNES, P.L. and HOLTFRETER, J. 1955. Directed movements and selective adhesion of embryonic amphibian cells. *J. Exp. Zool.* 128 : 53-120.

TRINKAUS, J.P. 1963. The cellular basis of *Fundulus* epiboly. Adhesivity of blastula and gastrula cells in culture. *Develop. Biol.* 7 : 513-532.

TRINKAUS, J.P. 1966. *In:* Major Problems in Developmental Biology. (ed.) M. Locke. Academic Press, New York. pp 125-176.

TYLER, A. 1940. Agglutination of sea urchin eggs by means of a substance extracted from the eggs. *P.N.A.S.* 26 : 249-256.

TYLER, A. 1942. Specific interacting substances of egg and sperm. *West. J. Surg. Obst. Gynecol.* 50 : 126-138.

VASSAR, P.S. 1963. The electric charge density of human tumour cell surfaces. *Lab. Investig.* 12 : 1072-1077.

VERWEY, E.J.W. and OVERBEEK, J.Th.G. 1948. *In:* Theory of The Stability of Lyophobic Colloids. Elsevier, Amsterdam.

VOGT, W. 1929. Gestaltungsanalyse am amphibienkien mit ortlicher Vitalfarbung. II. Teil: gastrulation und mesodermbildung bei Urodelen und Anuran. *Arch. Entwicklungsmech. Organ.*

120 : 384-706.

WALLACH, D. and EYLAR, E.H. 1961. Sialic acid in the cellular membranes of Ehrlich ascites and carcinoma cells. *Biochem. Biophys. Acta* 52 : 594-596.

WARD, P.D., and AMBROSE, E.J. 1969. Electrophoretic and chemical characterization of the charged groups at the surface of the murine CL_3 ascites leukaemia cells. *J. Cell Science* 4 : 289-298.

WARREN, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* 234 : 1971-1975.

WEISS, L. 1963. The mammalian tissue cell surface. *Biochem. Soc. Symp.* 22 : 32-50.

WEISS, L. 1965. Studies on cell deformability. I. Effect of surface charge. *J. Cell Biol.* 26 : 735-739.

WEISS, L. 1968. Studies on cell adhesion in tissue culture. IX. Electrophoretic mobility and contact phenomena. *Exp. Cell Res.* 51 : 609-625.

WEISS, L. and MAYHEW, E. 1966. The presence of ribonucleic acid within the peripheral zones of two types of mammalian cell. *J. Cell Physiol.* 68 : 345-359.

WEISS, L., and MAYHEW, E. 1967. Ribonucleic acid within the cellular peripheral zone and the binding of calcium to ionogenic sites. *J. Cell Physiol.* 69 : 281-291.

WEISS, P. 1950. Perspectives in the field of morphogenesis. *Quart. Rev. Biol.* 25 : 177.

WILKINS, D.J., OTTEWILL, R.H., and BANGHAM, A.D. 1962. On the flocculation of sheep leucocytes. I. Electrophoretic studies. *J. Theor. Biol.* 2 : 165-175.

ZWILLING, E. 1954. Dissociation of chick embryo cells by means of a chelating agent. *Science* 120 : 219.